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CONTRACT NUMBER DAMD17-96-C-6045

TITLE: Absorbable Gels for Modulated Bioavailability of Vaccines

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REPORT DATE: November 1996

TYPE OF REPORT: Final, Phase I

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	November 1996	Final, Phase I (15 Mar 96 - 26 Oct 96)

4. TITLE AND SUBTITLE
Absorbable Gels for Modulated Bioavailability of Vaccines

5. FUNDING NUMBERS
DAMD17-96-C-6045

6. AUTHOR(S)
Shalaby W. Shalaby, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (specific authority). Other requests for this document shall be referred to Commander, U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RMI-S, Fort Detrick, Frederick, MD 21702-5012.

12b. DISTRIBUTION CODE
19 DEC 1996

13. ABSTRACT *(Maximum 200*

The research question addressed in Phase I was: Would selected members of a novel family of absorbable copolyester gel formers be suitable as safe carriers for the development of a single-dose, controlled-release, injectable formulation of vaccines, using ricin A-chain (RAC) as a model, to provide timely, high antibody response, and prolonged immunity against respective pathogens?

Available Phase I results **do not only fulfill the initially sought positive response to the research question, but also provided us with the knowledge that** a single-shot, absorbable subcutaneous (*sc*) formulation, GF-II, exhibits potentially unique *in vivo* performance as it comprises a microparticulate cation-exchanger. **In addition** to the planned *sc* study of Phase I, preliminary studies were conducted in mice to explore the possibility of intranasal (*in*) immunization. Analysis of available data gave us the incentive to propose the development of a fluid, tissue-adhering, gel-forming formulation comprising a microparticulate ion-exchanger for *in* vaccination. Collectively, the development of an absorbable gel-forming controlled system of RAC and potentially other vaccines is feasible and plans for a successful execution of Phase II are in place.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Vaccine, Ricin, Injectable, Gel, Antibody, Absorbable, Biodegradable, Polymer	52
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

Attachment J.5.

FOREWORD

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T.W. Shalaby

Principal Investigator's Signature

10/14/96

Date

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A. INTRODUCTION

A.1. Background to Phase I Study--Growing interest in vaccination and diversity of new vaccines while relying on a would-be, out-dated, multiple-dose mode of administration and associated compromise of clinical effectiveness, evoked the search for a novel, single-dose (or single-shot), highly efficacious, injectable controlled delivery system. This, and the impressive advancement in the synthesis of absorbable polymers as potential matrices of traditional and less traditional drugs directed our attention to the identification of an absorbable polymer matrix that (1) is compatible with different vaccines; (2) maintains viability of live vaccines and/or retains their full activity; (3) provides an environment that assures vaccine stability under normal storage conditions; (4) does not alter the vaccine activity, as will be encountered in solution-based systems; (5) can be converted to single-dose parenteral formulations with predicable, reproducible efficacy; (6) can potentially be adapted for the development of oral and/or intranasal formulations for one-step immunization; (7) can provide vaccine formulation with minimum vaccine loading to develop timely antibody response and support exceptional persistence of protection against respective pathogens; (8) can be easily administered without the need to reconstitute; (9) is stable under normal storage and use conditions; (10) can be easily incorporated in the carrier and precisely administered; and (11) can be used as a carrier of live vaccine without compromising its activity.

A.2. Technical Objectives of Phase I--The research question addressed in Phase I was:

> **Would selected members of a novel family of absorbable copolyester gel formers be suitable as safe carriers for the development of a single-dose, controlled-release, injectable formulation of vaccines, using ricin A-chain (RAC) as a model, to provide timely, high antibody response, and prolonged immunity against respective pathogens?**

Accordingly, the primary technical objectives of Phase I were to: (1) review of available in-house data on the performance of different members of the gel-former family as carriers for the controlled release of antibiotics, polypeptides, and bovine serum albumin and select the most promising compositions for the RAC vaccine formulation; (2) Synthesis and characterization of needed polymeric components of the selected composition; (3) aseptic preparation and characterization of necessary formulations; (4) determination of the *in vitro* release profile of different formulations; (5) completion of a statistically designed immunization study in mice and determination of the antibody response; (6) use of immunized mice to assess the persistence of protection against an aerosol RAC challenge; and (7) analysis of the *in vitro* and *in vivo* results with correlation to matrix compositions to determine plans for Phase II study.

A.3. Related Work to Phase I Study

A.3.1. Vaccines and Contemporary Needs for Their Controlled Delivery--Immunization or treatment of animals or humans for making them immune to subsequent attack by a particular pathogen is usually accomplished by the oral or parenteral administration (or vaccination) of pharmaceutical preparations of a specific vaccine. The latter may contain a sublethal dose of a killed microorganism, living attenuated organism, living fully virulent organism, or pertinent recombinant DNA product. In order to achieve prolonged immunity, more than a single dose of the vaccine is usually needed. For many patients, and particularly in children, oral vaccination is a preferred route of administration. Nonetheless, injections of an aqueous formulation of vaccine is quite common. In both routes of administration, booster doses are required to develop sufficient immune response and achieve prolonged immunity. However, it has been demonstrated by a number of investigators that the use of a single dose injection of vaccine in controlled release systems is more efficacious than the traditional multidose of injectable, aqueous vaccine

solutions (Edelman et al., 1993; Eldrige et al., 1990; O'Hagan et al., 1993; Wise et al., 1987; Yan et al., 1995b). Meanwhile, Bowersock et al. (1994), in their study of the oral vaccination of cattle, made a strong argument for the advantages of using a controlled delivery system over traditional parenteral administration. In this particular study, poly(methacrylic acid) hydrogels were used for oral administration of bacterial exotoxins as vaccine for bacterial pneumonia over a five-day period. The study showed that hydrogels can deliver antigens orally to ruminants resulting in enhanced immunity at distant mucosal sites and reduced pulmonary lesions in calves challenged by a virulent respiratory bacterium.

A useful strategy to maximize the efficacy of vaccines without the need for controlled delivery systems, is the use of adjuvants. This approach has shown some promise in increasing the efficacy of vaccines such as anthrax (Ivins et al., 1990, 1992). However, the use of adjuvants still requires a multiple dose administration of the vaccine, a situation that can only be eliminated through the use of controlled delivery systems.

In a recent review on the development of oral vaccines to stimulate mucosal and systemic immunity, Shalaby (1995a) discussed novel strategies entailing the use of polymeric carriers to facilitate uptake by M-cells and prolong antigen presentation and stimulation of the Peyer's patches. He then suggested that improving the efficacy of oral vaccines may have direct application in the treatment of autoimmune diseases by facilitating the induction of oral tolerance.

Noticeable interest in the controlled delivery of vaccines developed amid reported, pertinent successes with traditional synthetic drugs (Dunn & Ottenbrite, 1991; Park et al., 1993; Shalaby et al., 1994), and particularly antiviral agents (Gangemi et al., 1986; Kende et al., 1988). Most pertinent to the proposed program is the use of biodegradable matrices for the controlled release of vaccines (Edelman et al., 1993; O'Hagan et al., 1993; Eldrige et al., 1990; Wise et al., 1987; Yan et al., 1995a). More specifically, microspheres made of lactide/glycolide copolymers were used for the controlled release of (1) orally administered vaccine, targeted to the Peyer's patches (Eldrige, 1990), and (2) ricin toxoid vaccine as injectable formulations (Yan et al., 1995a & b).

A.3.2. Absorbable Polymers for Controlled Drug Release--Absorbable (or often called biodegradable) polymers have been used clinically in sutures and allied surgical augmentation devices to eliminate the need for a second surgery to remove functionally equivalent non-absorbable devices (Schmitt, 1976; Shalaby, 1988). Although most of these devices were designed for repairing soft tissues, interest in using such transient devices, with or without biologically active components, in dental and orthopedic applications has grown significantly over the past few years (Damani, 1993; Dunn, et al., 1994, 1990; Schmitt, 1976; Shalaby et al., 1992b).

A.3.3. Absorbable Hydrogel-Forming Carriers for Controlled Drug Delivery--Growing interest in developing absorbable pharmaceutical and surgical products which degrade in the biological environment to safe by-products and leave no residual mass at the application site (Shalaby, 1985a, b & c, 1988, 1991a & b, 1992a, 1994a, b & c; Shalaby & Shalaby, 1993), justified the search for novel, absorbable gels. In a recent disclosure (Shalaby, 1995), novel gel formers were described to be based on absorbable copolymers which, upon hydration, result in hydrogels that are stabilized by pseudo-crosslinks provided by hydrophobic polyester components covalently linked to hydrophilic ones made of pharmaceutically acceptable polymer, such as polyoxyethylene. The polyester component is made of safe monomers, such as p-dioxanone, ϵ-caprolactone, glycolide, lactide, and mixtures thereof. Contrary to a related study (Dunn, et al., 1990), which describes *in-situ* formation of biodegradable, microporous, solid implants in a living body through coagulation of a solution of a polymer in an organic solvent such as N-methyl-2-pyrrolidine, the new hydrogel formers do not require the use of solvents. Such solvents did include low

molecular organic ones that can migrate from the application site and cause damage to living tissue, such as cell dehydration and necrosis. Equally important is the fact that previously known systems are solid implants which can elicit mechanical incompatibility and, hence, patient discomfort as compared with the new compliant, swollen, mechanically compatible hydrogels (Shalaby, 1995). Meanwhile, potential applications of the *in-situ*-forming implants, and the more recent gel-formers, have been described to entail their use for tissue regeneration and release of growth factors (Dunn et al., 1994; Shalaby et al., 1995). Depending on the composition of the gel-formers used in the present study, these absorbable matrices can be used for the controlled release of antibiotics over a period of 1 to 6 weeks (Shalaby, 1995).

B. MATERIALS and METHODS

B.1. Material--Monomers and chemical reagents used in this study are dl-lactide (Purac, Inc.) and glycolide (NORAMCO, Inc.), polyethylene glycol 400 (Aldrich Chemical), glycolic acid (Aldrich), and stannous octoate (Sigma Chemical). Ricin A-Chain was used in the form of commercial protein (RAC-L) obtained from Inland Laboratories. This is a solution of the protein in a buffered solution containing glycerol. The RAC-L was purified at USAMRIID by dialysis and then lyophilized. Two protein assay kits, BCA and Micro-BCA were purchased from Pierce Laboratories.

B.2. Methods--The polymers needed for the study were prepared according to the methods described by Shalaby (1996). The polymer characterization was conducted using a (1) Waters Associates GPC for molecular weight determination and purity; (2) Brucker-300 MHz NMR and Perkin-Elmer Paragon FTIR (Paragon 1000) for chemical structure verification; (3) Perkin Elmer DSC-600 for thermal properties; and (4) an LS particle size analyzer (Fluid Energy Aljet) for particle size distribution. The gel formulations were prepared in a laminar flow hood using a PMI-made micromixer. Homogeneity of the formulation is checked by optical microscopy. *In vitro* release of the ricin A-chain from different formulations was conducted using a phosphate buffer at pH 7.2 and 37°C in a shaker incubator. The released RAC was determined by a total protein assay using a BCA and Micro-BCA kit (see Appendix A), and a 6-550 UV/vis Sargent Welch spectrophotometer to measure absorbance.

The formulations were administered to mice both *sc* and *in* as described in the animal protocol (see Appendix B). The serum antibody titer was determined using ELISA; ricin A-chain and ricin toxoid were used as the fixed antigens in Poly-Med (see Appendix C) and USAMRIID conducted experiments, respectively.

Additional details on the methods used in this study were also given in the Experimental Results & Discussion Section.

C. EXPERIMENTAL RESULTS & DISCUSSION

C.1. Summary of Available Results and Significance--In concert with **meeting the major criteria for a successful Phase I study**, available results on *sc* administered active formulations do verify that (1) gel formulations can be easily prepared and appear suitable for scale-up; (2) one *sc* formulation is capable of releasing sufficient amounts of RAC to elicit IgG formation at high levels over a period of 4-20 weeks; (3) one formulation provides persistent antibody response at least 6 weeks post-immunization; and (4) a correlation can be established between IgG formation and the composition of the polymeric carriers. Available Phase I results **do not only fulfill the initially sought criteria for a successful Phase I study, but also provided us with the knowledge that** (1) a single-shot, absorbable *sc* formulation, GF-II, exhibits potentially unique *in vivo* performance as it comprises a microparticulate cation-exchanger; (2) upon comparing commercial RAC solution (RAC-L) with GF-II, the latter elicits a more gradual antibody

response that peaks at 10 weeks and exceeds a fast-decaying, initially higher response to RAC-L; (3) using ricin toxoid as a fixed antigen in ELISA evaluation of the antibody titer at USAMRIID at 6, 10, and 20 weeks for *sc* post-immunization indicate that the IgG titer for GF-II continued to increase significantly, while that of RAC-L declined considerably beyond 10 weeks; (4) in terms of antibody response, GF-II is associated with higher durability over the 10- to 20-week period; and (5) GF-II elicits a higher response of IgG-2A than RAC-L at 6 weeks. **In addition** to the planned *sc* study of Phase I, preliminary studies were initiated in mice to explore the possibility of administering, **intranasally** (*in*), a low viscosity gel-former (GF-III) and comparing its efficacy for *in* immunization with that of RAC-L. However, available data indicate that (1) mice are not a suitable model for *in* delivery; (2) *in* administration of RAC-L can be fatal; and (3) more rigorously tailored controlled release formulations are needed for this route of administration. Intranasal challenge of the different groups suggest that during the processing of RAC-L to incorporate into the gel-formulations, the protective segment of the protein has been altered and immunized mice were not resistant to the ricin toxoid challenge. **Collectively**, it can be concluded that the gel-former technology (1) is useful for the development of *sc* RAC formulation for one-shot immunization; (2) needs to entail optimization of RAC processing to prevent suspected alteration of its protective component in the course of its incorporation into the gel-former; (3) can be extended for use with other vaccines of military and non-military significance; (4) may provide a new platform based on the use of ionic microparticles and nanoparticles for developing unique delivery systems for highly potent proteins, including vaccines; and (5) may provide a highly successful means for *in* delivery of low doses of a variety of potent vaccines, with careful manipulation of the gel-formers and the ion exchanger.

C.2. Accomplishments of Phase I Tasks and Beyond--These accomplishments are outlined below in chronological order as documented in the first five monthly reports and the last few weeks. Additional data will be included in the addendum to this report (October 25, 1996).

C.2.1. Processing of Commercial Ricin A-Chain--As per the recommendation of the Contracting Officer (CO) the original plans to use Ricin Toxoid (RT) were changed to substitute the latter with Ricin A-Chain (RAC). To render the commercially available solution of RAC suitable for an unhydrous delivery system, it was dialyzed (to remove the buffer system and glycerol) and then lyophilized. Such processing may have compromised, to a limited extent, RAC activity, but it was decided to use it for timely execution of the study. A study is being conducted to test this postulate.

C.2.2. *In vitro*-Release Study of RAC from Candidate Gel-Formulations and Selection of a Gel-Former for the *In Vivo* Study--To identify a sensitive analytical procedure for determining exceptionally low concentrations of Ricin A-Chain during the *in vitro* release studies, three established analytical methods were evaluated using a diluted sample of the commercial RAC solution (RAC-L). These methods are (1) an HPLC procedure using a C18 column and an acetonitrile/trifluoroacetic acid as the mobile phase; (2) the bicinchoninic acid (BCA) assay; and (3) the micro-BCA assay. The first two methods were found to be suited for high RAC concentrations of 1.5 mg/ml and 100 μg/ml, respectively. However, the micro-BCA was found useful for monitoring concentrations of 5 μg/ml. Thus, the latter method was selected for future use in comparative *in vitro* release studies of the final set of gel forming candidates. The identification of the final set of candidates was realized by examining several gel-formers (1) in a brief mass loss or absorption study to determine, semi-quantitatively, the relative absorbability of these gels; and (2) as vehicles for the controlled release of low- and high-molecular weight model proteins at 1% loading and determine their release profile.

In the comparative mass retention study (Table I), nine (GF10 to GF18) available gel-formers were loaded with insulin ($\sim$ 6 kDa) and bovine serum albumin (BSA, $\sim$ 60 kDa), and the release profiles of either proteins were determined in a phosphate buffered solution at 37°C and pH 7.2. Release data of this study

(Table I) using HPLC and BCA indicated that one of these compositions (GF11) and a new gel former (GF19) can be identified as being appropriate candidates for *in vitro* study of RAC. In a second set of *in vitro* release studies, RAC was used to determine which of the two liquid gel-formers (GF11 and GF19) are most suited for the animal study. However, due to interference of the polyethylene glycol component of the gel-formers with micro-BCA, the comparative results were valued as being semi-quantitative. It was possible, however, to select GF19 as the choice system for the *in vivo* study. This selection was consistent with our prediction of GF19 absorbability rating (Table I) based on credible compositional criteria. This was later denoted as GF-I and subsequently used to prepare GF-II. During this final selection process, an *in vitro* protocol was developed to monitor the release of RAC from the two final gel formers (GF11 and GF19). A phosphate buffer at 37°C and pH 7.2 was used for treating the gel formers containing RAC in a shaker incubator. A micro-BCA assay was used to determine the release of RAC into the buffered medium over a 4-week period. It was also determined that of the gel-forming components, only the polyethylene glycol contributed to the optical density of the analytical reagent. Semi-quantitative analysis of the release data and review of earlier release data with model proteins supported our selection of GF19 (or GF-I as referred to later) for the *in vivo* release study.

Table I--*In Vitro* Release Data of Gel-Forming Compositions
in Phosphate Buffer @ 37°C and pH 7.2

No.	Composition[a] Components (%)	Absorption Rating(*b*)	% Release of Insulin		% Release of BSA	
			At 3 days	At 10 days	4.2 days	19 days
GF10	P_1(60), P_3(40)	4	35.7	44.9	--	--
GF11	P_4(91), P3(9)	2	13.2	29.9	--	--
GF12	P_5(70), P_3(30)	1	0	0	--	--
GF13	P_6(40), P_7(60)	3	29.5	48.1	--	--
GF14	P_8(40), P_7(60)	3	18.3	26.4	--	--
GF15	P_9(40), P_7(60)	3	17.0	24.7	--	--
GF16	P_{10}(50), P_7(50)	2	10.0	10.0	--	--
GF17	P_1(80), P_3(20)	3	--	--	16.5	21.7
GF18	P_2(80), P_3(20)	5	--	--	34.8	40.6
GF19	P_1(62), P_2(19), P_3(19)	2[c]	--	--	--	--

(a) P_1 = (20/80)/(60/40) PEG-400/(L/G) P_2 = (30/70)/(60/40) PEG-400/(L/G)
P_3 = (85/15)/(60/40) PEG-400/(L/G) P_4 = (30/70)/(80/20) PEG-600/(L/G)
P_5 = (30/70)/(80/20) PEG-1000/(L/G) P_6 = (20/80)/(92/8) PEG-1000/(CL/G)
P_7 = (85/15)/(92/8) PEG-400/(CL/G) P_8 = (30/70)/(92/8) PEG-1000/(CL/G)
P_9 = (20/80)/(92/8) PEG-400/(CL/G) P_{10} = (30/70)/(87/13) PEG-1000/(CL/G)
(b) Absorption rating is based on subjective mass retention during a 2-week period, with a rating of 1 and 5 reflecting minimum and maximum absorption, respectively. (c) Projected rating.

C.2.3. Preparation of GF-I--To prepare the new GF19 (later known as GF-I) composition, P_1, P_2, and P_3 were made (Table I). These are A-B-A block copolymers of polyethylene glycol 400 and 60/40 dl-lactide/glycolide with A/B ratio ranging from 15 to 80 percent by weight. The syntheses of these

copolymers were conducted in the presence of stannous octoate as initiator. The resulting polymers P_{-1}, P_{-2}, and P_{-3} were characterized by IR, NMR, and GPC to confirm their chemical identity, purity and determine their molecular weight (relative to a polystyrene standard) and polydispersity.

C.2.4. Preparation of the Cation-Exchanger--The cation-exchanger or microparticulate carrier (CE) was made by the ring opening polymerization of glycolide using glycolic acid as the initiator and a catalytic amount of stannous octoate. Practically monomer-free polymer was isolated, ground, micronized, and then characterized by DSC and IR. Particle size distribution of the micronized CE was also determined.

C.2.5. Development and Use of a Micronization Protocol for CE--The polymer was first ground into particles having a diameter of about 150-200 μ using a Wiley mill. The resulting granules were micronized by roll milling in an inert medium or by jet milling. The latter technique provided a more controlled particle size distribution. Depending on the processing conditions, three sets of micronized particles having an average diameter of 14, 6 and 4 μ were produced. The latter particle size was considered optimal for the RAC study. In September 1996, a microjet mill was purchased and is presently used to produce micronized CE at the desired diameter using high purity, dry nitrogen to provide necessary high pressure clean gas flow for operating the mill.

C.2.6. Preparation of Two Selected Formulations for the *In Vivo* Studies--Lyophilized RAC in deionized, sterile water was mixed mechanically with micronized CE (using a specially constructed syringe micromixer). Water was then evaporated (in a sterile field) from the micromixer under reduced pressure at room temperature. The dry, coated CE was then mixed with the gel former in a sterile field and the mixture was prepared for use in a syringe for injecting the formulation.

C.2.7. Revision of the Animal Protocol for *sc* Administration of RAC Formulation in Mice and Conducting the *In Vivo* Studies--Following a careful review of the animal protocol, a number of adjustments were made to maximize the effectiveness of the study. These included (1) the use of a gel-former placebo to determine any effect on antibody production or any symptomatic reaction to its administration; (2) the use of an intermediate dose of 20 μg/animal (not the low 10 μg dose/animal); (3) using a one shot RAC from aqueous solution; and (4) withdrawing blood samples for each set at 0 time. The finalized experimental plans are summarized in Table II.

A total of 90 mice were used in the study for the subcutaneous (*sc*) administration. Two of these were used to master the administration techniques and 88 were dedicated for the actual study. This was initiated on May 2, 1996. Sufficient number of blood samples for each set of animals were withdrawn and the respective sera were frozen at -70°C awaiting their use in the ELISA assay in the next reporting period.

Table II. Finalized Experimental Plans for the *sc* Delivery Systems

Segments	Control	Placebo I	Formulation I	Formulation II
Formulation for Subcutaneous Administration				
•Polymer	None	190 μl Gel-former (GF) + mg	200 μl GF	190 μ GF + 10 mg MC
•Ricin A-Chain (RAC)	20 μg RAC + 200 μl H_2O	Microparticulate carrier (MC) None	20 μg RAC	20 μg RAC
Determination of Antibody titer (a)	<----------0, 4, 6, 8, & 10 weeks---------->			
Immunization and Persistence of Protection (b)				
• Determination of antibody titer (a)	Immediately prior to transfer for challenge.	N/A	Immediately prior to transfer for challenge.	Immediately prior to transfer for challenge.
• Post-immunization challenge (c)	6 & 8 weeks	N/A	6 & 10 weeks	6 & 10 weeks

(a) Each experiment will be run in a set of seven animals. The titer determination periods were changed to 4, 6, 10, 14, 20, and 24 weeks. (b) Each experiment will run in a set of ten animals. (c) Post-immunization challenge will be conducted at Fort Detrick--the challenge periods were changed and the first challenge would have been conducted at 20.5 weeks; the second will take place at 24 weeks.

C.2.8. Development of a modified ELISA Assay of Mouse Serum for Anti Ricin A-Chain (Anti-RAC) and Its First Application to 4-week *sc* Sera--Using the 4-week sera, the anti-RAC antibodies of all samples were determined using the newly developed ELISA assay. In each set of the ELISA testing, five plates were run, the titer-plate and four unknown groups. The unknown groups contained serum from six of the antibody mice that were run in duplicate. Each plate contained positive and negative control dilutions. Two wells on each plate were sacrificed in order to simultaneously run a positive and negative control to increase confidence in the results indicating assay uniformity. The titer plate demonstrated a normal serological response and the high concentration of the positive antibody appears to inhibit the binding. At dilution of 1:800 and higher, the response was practically as expected. The end point of the unknown serum was defined as the least dilution at which the OD was twice that of the background. Analysis of data obtained so far suggests that (1) although the new ELISA assay can be used to monitor the formation of anti-RAC antibodies in treated animals, further fine-tuning of the technique

may be required; (2) the placebo gel formulation does not elicit anti-RAC antibody response; (3) in animals which were just injected with the active or inactive formulations, no anti-RAC antibodies could be detected in their sera when samples were taken prior to these injections; and (4) at the 4-week period, mice treated with the commercial aqueous RAC and gel formulation II showed substantial response and less pronounced effect with the gel formulation I, namely in sets of six mice, 6/6, 5/6, and 3/6 mice, indicated positive responses, respectively.

C.2.9. Fine-Tuning the Modified ELISA and Its Application in the 6 and 10 Week Antibody Titer Determination--Study mice were bled and processed sera were assayed for antibody response as noted for the 6-week set of mice. Preliminary ELISA data indicate that GF-II performance continued to show progressive increase in antibody response to a level that is at least comparable to that of the liquid RAC. It should be noted that GF-II showed moderate response at 4 weeks as compared to liquid RAC and edged upward at 6 weeks to trail liquid RAC performance. The ELISA assay data may reflect a **persistence of the antibody response to GF-II and commencement of a decline in the response to RAC-L. Although these observations may be considered tentative, they are in tandem with our original hypothesis**. Meanwhile, the antibody response to GF-I was practically absent at 14 weeks.

C.2.10. Antibody Titer Determination of 14- and 20-Week *sc* Sera--The antibody titer data indicate the changes in the titer values for RAC-L, GF-I, and GF-II were consistent with the trend observed about the 10-week period, as shown in Figure 1 and Table III. Specifically, (1) no significant response was elicited by GF-I at 14-20 weeks; (2) response to both RAC-L and GF-II continued to decrease but the rate of change for RAC-L was generally more than that associated with GF-II; and response at 20 weeks remained higher for GF-II as compared with RAC-L.

Table III. ELISA Antibody Titer Data Determined by Poly-Med*

	Geometric Mean Titer				
Site	For Subcutaneous Administration			For Intranasal Administration	
Formulation	RAC-L	GF-II	GF-I	RAC-L	GF-III
Immunization Period					
4	5701	459	63	--	--
6	1425	1055	63	112	50
10	1269	1392	63	--	--
12	--	--	--	100	50
14	712	1212	50	--	--
20	400	566	50	--	--

* RAC-L was used as the fixed antigen.

C.2.11. Antibody Titer Study of 6, 10, and 20 Week *sc* Sera at USAMRIID and Comparison with Respective Data Obtained by Poly-Med--Results due to independent sets of experiments pursued by Dr.

M. Kende of USAMRIID on *sc* sera obtained at 6, 10, and 20 weeks (the 4-week sera were not available for USAMRIID evaluation) using ricin toxoid as the fixed antigen indicate that (1) the antibody titer values for IgG obtained by PMI are generally lower than those recorded at USAMRIID; (2) at 6 and 10 weeks, the IgG titer decreased in the following order--RAC-L > GF-II > GF-I; (3) at 6 weeks the IgG-1 decreased in the order of GF-II > RAC-L > GF-I; (4) at 10 weeks the IgG-1 titer decreased in order of RAC-L > to GF-II > GF-I; (5) at 6 weeks, IgG-2A titer for RAC-L and GF-I were comparable and significantly less than that of GF-II; (6) at 10 weeks, the IgG-2A titer decreased in the order of RAC-L > GF-II> GF-I; and (7) the IgG titer due to GF-II increased significantly from 6 to 20 weeks, while that of RAC declined considerably beyond 10 weeks. These data are illustrated in Figures 1 to 6 and Tables III and IV. A possible explanation of the high titer values obtained by USAMRIID is the presence of B-chain impurities in ricin A-chain formulations, whose antibody response can only be detected by ricin toxoid as the fixed antigen.

ELISA data for sera of immunized mice obtained at 6, 10 and 20 weeks, previously generated by PMI (using ricin A-chain as the fixed antigen), were consistent with data for the same samples obtained at USAMRIID (using the ricin toxoid as the fixed antigen) in terms of observed trends. Additionally, the net antibody responses were generally higher as determined by the USAMRIID assay. According to both the PMI and USAMRIID ELISA data, full protection of the 20.5-week *sc* immunized mice is expected for GF-II and RAC-L. However, as will be seen later in the challenge study, this is not the case.

Table IV. Antibody Titer Data Determined by ELISA at USAMRIID
for Subcutaneously Administered Formulations

	Geometric Mean Titer							
	For 6-Week Immunization			For 10-Week Immunization			For 20 Week Immunization	
Formulation	IgG	IgG-1	IgG-2A	IgG	IgG-1	IgG-2A	IgG	IgG-1
RAC-L	92,720	4,753	5	102,329	33,773	11	53,703	3,118
GF-II	19,013	10,495	10	50,933	7,687	10	102,329	4,570
GF-I	10,495	5,248	5	7,711	794	7	6,165	176

C.2.12. Analysis of 20-Week Antibody Titer Data of Different *sc* Administered RAC Systems--The systems in question are the commercial liquid RAC (RAC-L) gel-forming formulation (without GF-I) and with a cation-exchanger (GF-II). The geometric means derived from ELISA data of the three systems are illustrated in Figure 1, which indicates that (1) response to RAD-L was exceptionally high at 6 weeks, but decreased at a fast rate over the subsequent 14 weeks; (2) response to GF-I was low at 4 weeks and continued to decay to insignificant levels at 10 weeks; (3) GF-II elicited a moderate response at 4 weeks, which gradually increased and surpassed that of RAC-L at 10 weeks, then it maintained a significant, but decreasing level over the subsequent 10 weeks; and (4) at 20 weeks, GF-II maintained a stronger response than RAC-L.

In view of the data presented in Figure 1, it was postulated that (1) the generally low response due to GF-II as compared to that of RAC-L is related to partial loss of activity during processing, as in dialysis and lyophilization of RAC; (2) the low response to GF-II at 4 weeks may be related to the limited

availability of RAC because of the very nature of the controlled release system; and (3) **the superiority of GF-II to GF-I** clearly reflects the key **role that the microparticulate cation-exchanger might have played** toward the desirable performance of GF-II. To determine the viability of these postulates, (1) a brief pilot study on the effect of RAC processing conditions, tested in an aqueous carrier, on the antibody titer has been initiated and will be continued beyond the original conclusion date of Phase I; (2) a pilot study on the effect of the type and concentration of ion-exchanger on GF-II performance is recommended for investigation after the completion of Phase I.

The IgG, IgG-1 and IgG-2A antibody titer data obtained at USAMRIID recorded above, are presented graphically in Figures 2 and 4. Key features of the data in these figures are (1) the noticeably high titer value for IgG-2A associated with GF-II at 6 weeks; (2) the low IgG, IgG-1 and IgG-2A titer values of GF-II as compared to RAC-L, even though Poly-Med data reflect higher IgG values for GF-II at 10 weeks; and (3) the highest IgG and IgG-1 responses for GF-II as compared to those due to GF-I and RAC-L. It is likely that the high IgG-2A titer values at 6 weeks has a significant effect on the GF-II performance beyond 6 weeks. The IgG-2A data at 6 weeks can be a reflection of an IgG subclass difference associated with GF-II and RAC-L. This may lead to high neutralization efficacy (Carayanopoulos & Capra, 1993) and, hence, high protection.

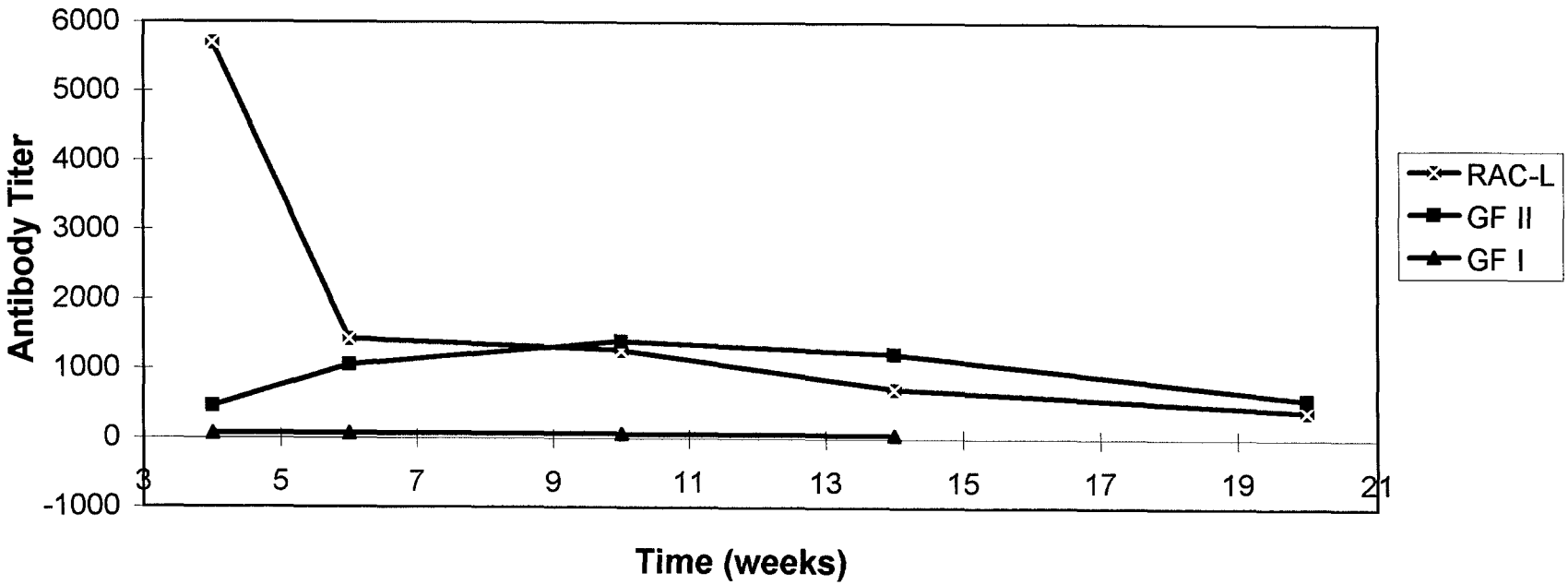

Figure 1. Antibody Titer For Ricin A chain from Subcutaneous Administration of Two Gel Formulations and a Liquid Control Determined by Poly-Med.

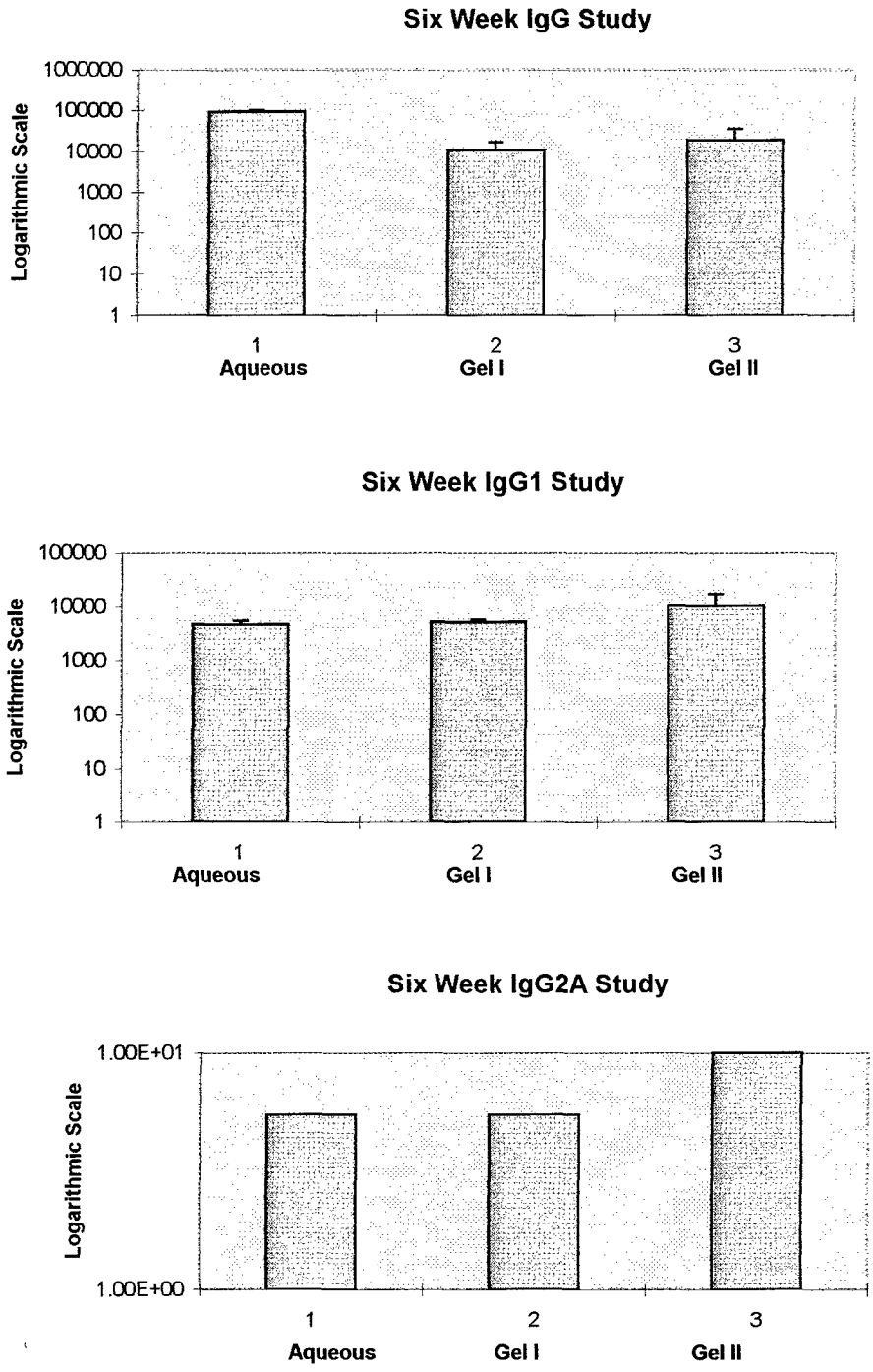

Figure 2. Six week antibody titer for subcutaneous administrated RAC formulation from ELISA testing at USAMRIID.

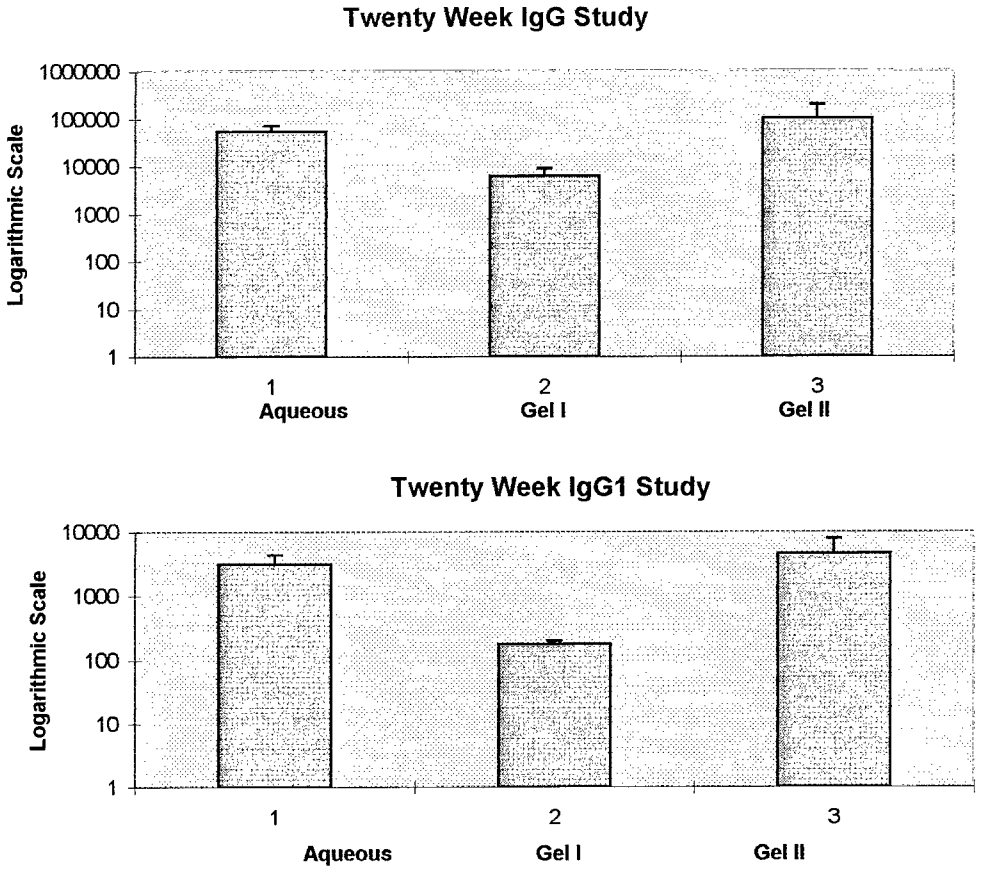

Figure 4. Twenty week antibody titer for subcutaneous administration RAC formulation from ELISA testing at USAMRIID.

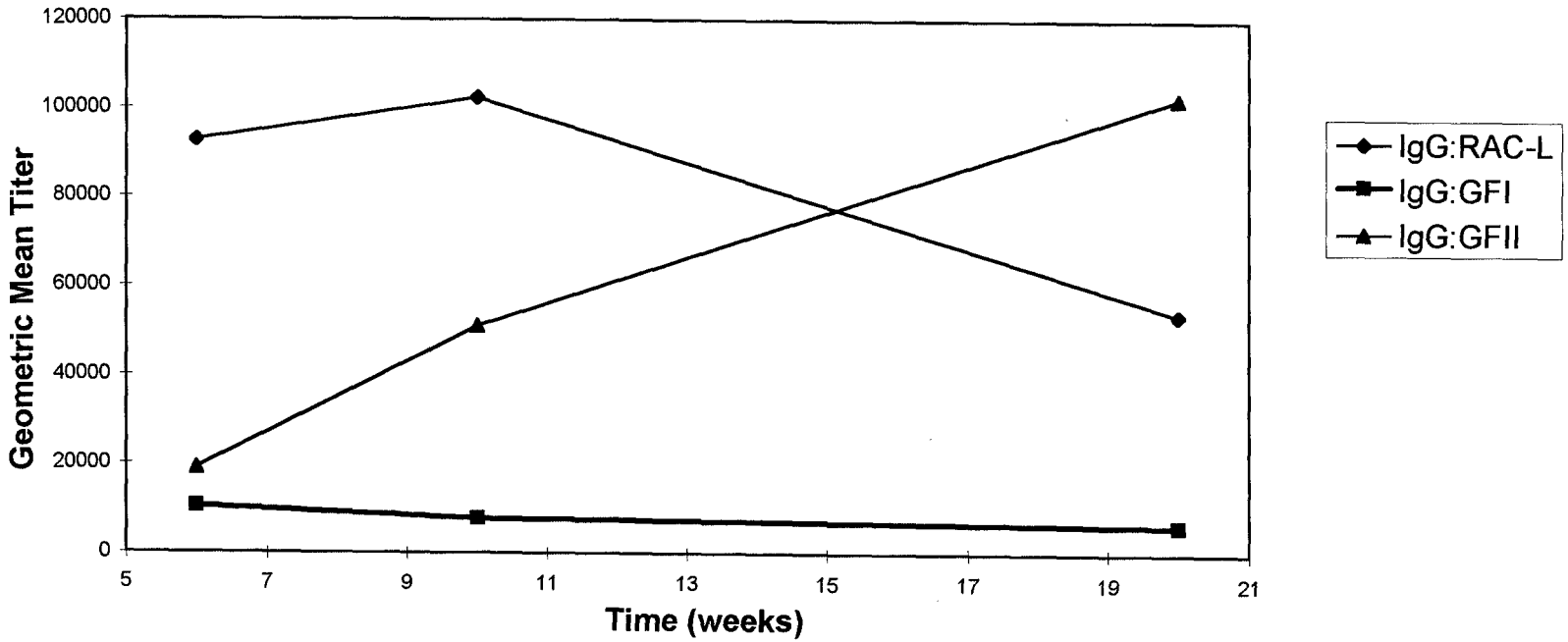

Figure 5. USAMRIID ELISA Antibody Titer of IgG for Subcutaneously Administered Formulations

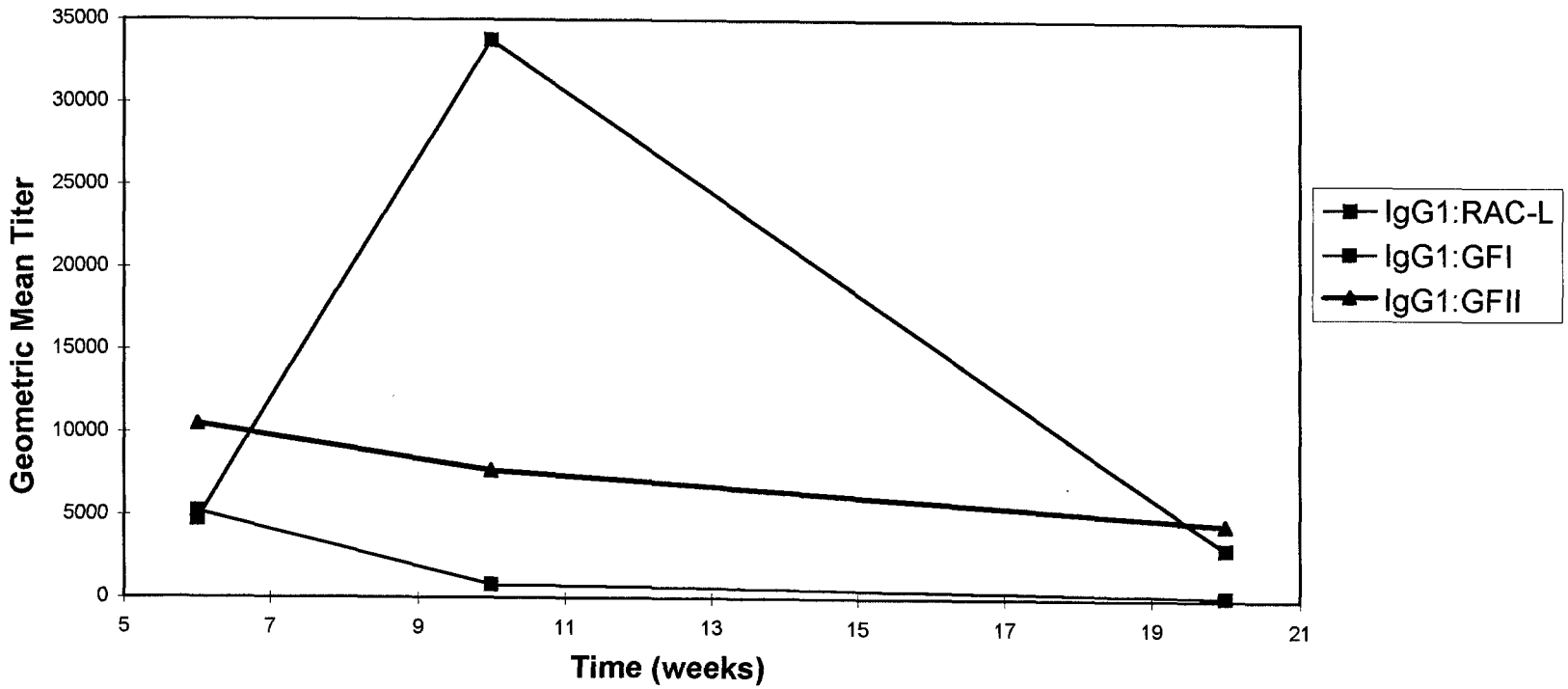

Figure 6. USAMRIID IgG1 ELISA Antibody Titer for Subcutaneously Administered Formulations

C.2.13. Intranasal Delivery of RAC: A Supplemental Segment to the Planned *sc* Study--In an effort to maximize the outcome of the Phase I study, a supplementary study was sought that calls for the intranasal delivery of a suitable gel formulation. For this, an amendment to the original animal protocol was prepared, submitted, and approved for implementation. This entails the use of 61 mice which will be allocated to the different segments of the study as in the original protocol with the exception of using only one new gel formulation (GF-III) instead the previous GF-I and GF-II. GF-III will consist of low viscosity matrix with CE carrier for the RAC. GF-III was administered into the two nostrils of the mice.

C.2.13.1. Development of Experimental Plans for the Intranasal Delivery Systems--The original protocol for the *sc* administration was adjusted to include a new segment for an intranasal administration of a new RAC formulation. This is expected to have RAC deposited on micronized CE in a low viscosity liquid gel-former that is expected to adhere to the nasal mucosa for a period of 12 to 36 hours. This is to allow free RAC and RAC coated microparticles to be incorporated into the mucosal membrane. An outline of the intranasal plans is given in Table V. The newly revised protocol was approved May 1, 1996. However, due to scheduling issues, the challenge periods were changed and the first challenge was conducted at 12.5 weeks.

Table V. Intranasal Immunization

Segments	Control	Placebo II	Formulation III
Formulation for Intranasal Administration			
• Polymer	None	47.5 μl GF + 2.5 mg MC	47.5 μl GF + 2.5 mg MC
• RAC	20 μg RAC + 200 μl H_20	None	20 μg RAC
Determination of Antibody Titer (a)	<----------0, 4, 6, 8, 10 weeks---------->		
Immunization and Persistence of Protection (b)			
• Determination of antibody titer	Immediately prior to transfer for challenge.	N/A	Immediately prior to transfer for challenge.
• Post-immunization challenge (c)	6 & 10 weeks	N/A	6 & 10 weeks

(a) Each experiment will be run in a set of seven animals. The titer determination periods were changed to 6, 12, and 16. (b) Each experiment will run in a set of ten animals. (c) Post-immunization challenge will be conducted at Fort Detrick--these dates were changed to have the first challenge at 12.5 and 16 weeks.

C.2.13.2. Preparation of Low Viscosity Gel-Former with Activated Cation-Exchanger--A new low-viscosity gel former was developed (after screening a few candidates) to meet the requirements of being (1) deliverable from a micro-syringe; (2) capable of wetting and spreading on the intranasal surface and

forming adherent hydrogel that resides at the administration site for one to three days; (3) a suitable matrix for the controlled release of free RAC molecules and phagocytosis of the microparticulate carriers of the RAC; and (4) undergoing minimum change in viscosity upon introducing microparticulates with RAC deposited on their ion-exchanging surface. The assembling of GF-III was done as reported earlier for GF-II. Sixty-one animals were divided into two groups, one for studying the antibody response (21 mice) and a second for the challenge study (40 mice). Of the 21 mice, a set of 7 was treated with the placebo of GF-III, commercial liquid RAC or active GF-III. The 40 mice of the challenge group were treated in two sets with liquid RAC or active GF-III, with the exception of two mice treated with RAC-L, which were sacrificed (due to noticeable weight loss) in the first week.

C.2.13.3. Pilot Test for Intranasal Immunization--Of the 65 mice procured, 3 were used to test the practicality of introducing 25μl of the new candidate gel formers. Based on gross observation, it was determined that a low-viscosity gel with activated microparticulate (GF-III containing a micronized polyglycolide with carboxylic-bearing surface as ion-exchanger) can be conveniently and precisely administered using a micro-syringe but only in 10-15μl quantities. Therefore, the animal protocol was amended to allow using the intended RAC dose in 10μl of carrier using a micro-syringe. And the rest of the animals for the *in* group were treated accordingly.

C.2.13.4. Antibody Titer Determination of Sera of Intranasally Treated Mice and Comparison with Pertinent *sc* Data--Preliminary ELISA data at 6 and 12 weeks do not reflect significant antibody response to intranasally administered GF-III. The difficulties encountered in administering the gel former to the exceptionally small mouse nostrils and likelihood of gel dislodgement may be related to noted results. Meanwhile, the response to RAC-L was far less pronounced than those noted for comparable periods for the *sc*-treated mice, as illustrated in Figure 4. Furthermore, two of the mice treated intranasally with RAC-L were sacrificed due to weight loss associated with respiratory complications. This may be related to possible "run-off" of the aqueous formulation to the lung. These results may lead one to conclude that: (1) the mouse animal model is not suitable for conducting intranasal administration of gel-formers and even aqueous solutions; (2) larger animals will be more suitable for intranasal administration of liquids and gel-formers; (3) the adhesion and rheological properties as well as hydrophilicity of the carrier must be adjusted to insure maximum mucosal coverage and high residence time about the administration site; (4) a specially designed nebulizer should be used for introducing the low viscosity formulation. It is important to note that the observed complications of the two animals, which had been treated with RAC-L, and comparison of the antibody titer in surviving mice (as compared to *sc*-treated ones), may suggest that highly potent vaccine in an aqueous carrier can migrate to the lung causing a highly undesirable local reactions; and part of the aqueous formulation may also be lost through migration to the gastrointestinal tract and reduces the vaccine's net activity. This behavior justifies the use of low-viscosity, highly adhering, gel-former with the vaccine immobilized temporarily on a suitable ion-exchanger.

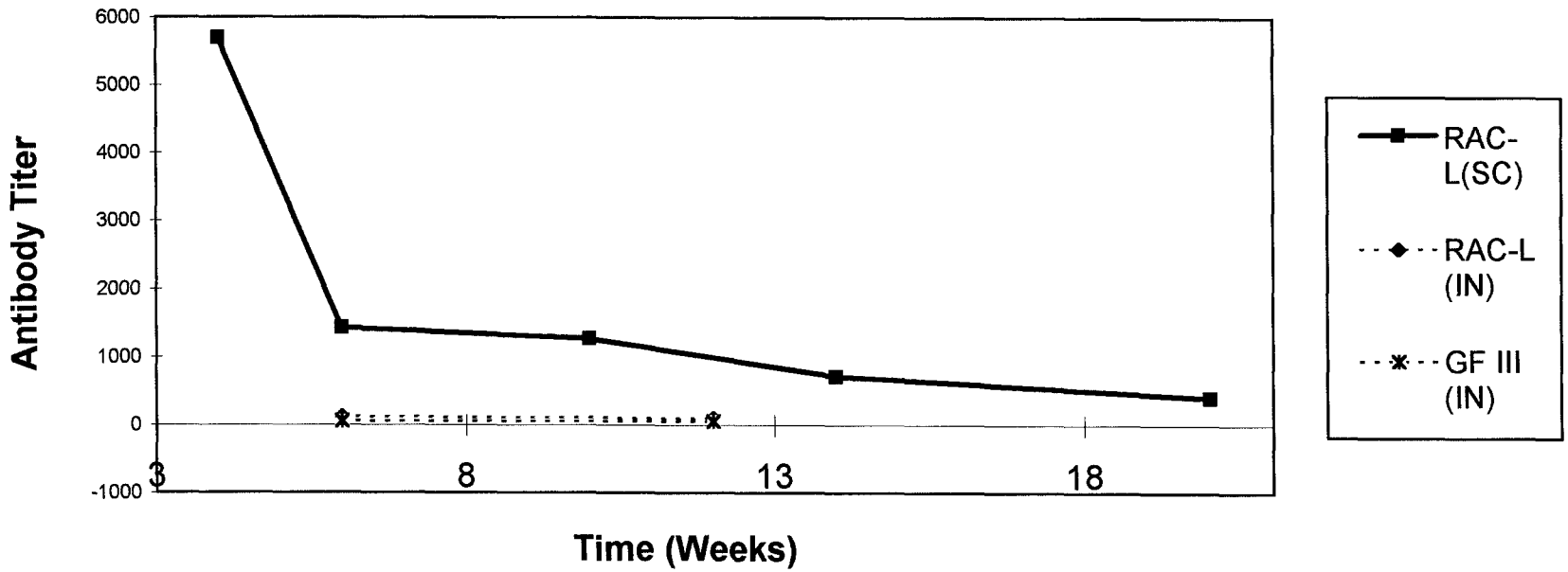

Figure 7. Comparison of Intranasal and Subcutaneous Titers from ELISA Testing by Poly-Med.

C.2.14. Intranasal Challenge of Immunized Mice and Control

Different groups of mice were challenged at USAMRIID with an intranasal aerosol of ricin toxoid. Description of these groups and survival data are given in Table VI. The survival data were recorded at 2 weeks post-challenge.

Table VI. Intranasal Challenge Data

Set A	*sc* Immunized Mice [a]			
Subset	Gel-II Placebo	RAC-L	Gel-II	Gel-I
No. of test animals	7	10	9	10
No. of survivals	0	10	0	0

Set B	*in* Immunized Mice [b]	
Subset	RAC-L	Gel-III
No. of test animals	9	10
No. of survivals	2	0

(a) Challenged at 20.5 week post-immunization.

(b) Challenged at 12.5 weeks post-immunization using the same control for the SC subset.

The results indicate that with the exception of the mice immunized subcutaneously with RAC-L and a few of the intranasally treated mice with RAC-L of the challenged subsets displayed no protection.

C.2.15. Effect of Dialysis and Lyophilization on Ricin A-Chain--The antibody titer data obtained by Poly-Med, which concur exceedingly well with independently generated data at USAMRIID (for 6, 10, and 20 weeks, only due to unavailability of the 4-week sera) on the same sera indicate that GF-II formulation based on lyophilized RAC represents a promising controlled delivery system for ricin A-chain. This is because it displayed a gradual increase in antibody titer which seemed to exceed that of the commercial solution (RAC-L) beyond the 10-weeks and maintained higher titer values through the balance of the 20-week post-immunization period. This is in contrast with the titer values of RAC-L which peaked at 4 weeks and decayed at a fast rate thereafter. However, the intranasal challenge, data of the different groups of mice suggest that the antibodies developed as a result of GF-II formulation do not protect the animal against the ricin toxoid. The inability of the antibodies associated with the GF-II immunization to neutralize the ricin toxoid may be related to changes in the ricin A-chain that took place during the **dialysis and lyophilization** processes (about 3 days), shipment (1 day), or attempted

reconstruction in sterile, deionized water (about 0.5 day), and drying on the cation-exchanger (about 1 day). These changes may have been caused by (1) exposure to room temperature for about 2 days during dialysis; (2) room temperature drying for about 24 hours of RAC deposited on the cation-exchanger; (3) conformational changes in the protein molecules during the lyophilization cycle; (4) hydrophobic or ionic associations of protein molecules during lyophilization, which alters the presentation of critical segments of the protein chain to the biological environment, leading to changes in the expected antigenic activity; and (5) irreversible molecular aggregation during lyophilization which compromises the bioavailability and/or impaired the antigenic activity of ricin A-chain as expected in the glycerol-stabilized form of the commercial buffered solution. The postulate of altered ricin A-chain activity during its purification processing can be appreciated if we acknowledge the documented variable effects of vaccine and protein processing conditions (Ahmed et al, 1974; Foxwell et al, 1985; Kume et al, 1977; Lemon et al, 1994; Lin & Kleven, 1987) on their activities. Following are a few illustrations of the complex effects on vaccine and protein activities.

In a study by Lemon et al (1994) on hepatitis A virus (HAV), it was noted that the virus is potentially sensitive to the removal of water, with a 0.44-2.28 log 10 reduction in infectivity accompanying the lyophilization of chloroform-extracted virus spiked into factor VIII. Lyophilization of Group-A streptococcal M protein was associated with the conversion of the dimeric form of both the 32kD PepM57 and 27kD PepM57 to the corresponding monomeric molecular forms, which, in turn, compromises the opsonic activity of the proteins (Khandke et al, 1991). Meanwhile, Ahmed et al (1974) reported that lyophilization of recombinant cholera toxin does not have any adverse effect on immunogenicity, as determined by antibody titer of sera from immunized mice. In a report by Lin and Kleven (1987) on the lyophilization of mycoplasma synoviae hemagglutination antigen (HA), it was suggested that an increase in the HA titer occurred following lyophilization. The authors also noted that their results disagree with those of Kume et al (1977) attributed to their high titer values to the stabilizing fluid used in their study. Lin and Kleven stressed the importance of the type of medium used in the reconstitution of lyophilized antigen on their performance.

Towards uncovering the cause of the disagreement between the RAC antibody titer and challenge data, the following brief experiment is being conducted: Two sets of 3 mice were separately injected with the same dose of commercial RAC-L and lyophilized vaccine that is reconstituted in the presence of glycerol. It is hoped that the antibody titer and challenge data for formulations devoid of any polymeric carrier, will provide some support or dispute our postulate on the impaired antigenicity of RAC during the preparation of the controlled release system.

C.3. Extended Activities and Concluding Results--The study period of Phase I was extended to October 26, 1996 (without additional funding) to allow prolonging the immunization studies and obtaining longer-term data. The remaining segments of Phase I study that will be completed in the sext several days are: (1) the second and final challenge study of *sc*-immunized mice and controls at 24 weeks; (2) the second and final challenge study of *in*-immunized mice and controls at 16 weeks; and (3) a brief pilot study on the effect of RAC processing conditioned on its effectiveness in eliciting antibody response and protecting *sc*-immunized mice when challenged intranasally with ricin toxoid.

C.4. Collective Analysis of Relevant Work and Design for Advanced Plans for Novel Vaccine Controlled Release Systems--Collective analysis of relevant work led us to the following conclusions: (1) There is a need for single-dose vaccine. (2) To achieve early response and prolonged immunity, controlled delivery of the vaccine is required for a single-dose vaccination. (3) Although there is alluring promise in orally administered vaccine for its pertinence submucosal stimulation (Shalaby, 1995a), new

delivery systems will be much easier to assess, initially, as injectable, intranasal formulations. (4) Absorbable polymers are excellent carriers for the study and development of a new controlled delivery for vaccines. (5) Although the drug delivery literature is rich with data on the use of absorbable microspheres, the difficulty associated with their manufacturing and sterilization, occasional problems with reproducibility and quality control, occasional problems with reproducible clinical efficacy, difficulties associated with formulation and injection, presence of undesirable processing impurities, potential migration from injection site, potential detrimental effects--as in the case of residual solvents on biological molecules; and possible mechanical incompatibility created a need for a new absorbable carrier. (6) Recently developed absorbable gel-forming polymer by Poly-Med, Inc., appears to meet key requirements for a vaccine carrier. These gel-formers do not entail a solvent as in the Atrix™ *in-situ*-forming, solid implants (Dunn et al., 1990, 1994), which can compromise vaccine efficacy and viability if live vaccine is used and patient safety. (7) Reported ability of the Atrix implants and Poly-Med gels to release growth factors and antibiotics, respectively, suggest strongly that the latter gels are capable of controlling the release of vaccines. (8) Ricin A-chain was the first chosen vaccine model, which was used successfully to study the new gel-forming polymers in Phase I of this program. (9) Liquid, injectable gel-formers were shown to provide different release profiles by incorporating limited amounts of an absorbable ion-exchange resin (Shalaby, 1996). The use of such a strategy with ricin to afford ionic binding to microparticulate solids has shown great promise for the ricin system and determining its broad applicability to other systems is a logical objective of Phase II.

D. CONCLUSIONS & RECOMMENDATIONS

Results of initially planned Phase I activities and additional segments introduced during the course of the original plans led to the following **conclusions**:

1. Two members of the novel absorbable gel-forming formulation (GF-I and GF-II) can be easily produced and used as carriers of the ricin A-chain (RAC).

2. The two gel formulations behave significantly different as controlled release carriers of RAC and this difference is due to the presence of a microparticulate cation-exchanger.

3. Without having the cation-exchanger as in GF-I, the antibody titer value of sera from immunized mice were lower at the early periods of immunization than those due to the commercial RAC (i.e., RAC-L) and cation-exchanger-bearing gel former GF-II. In addition, antibody titer due to GF-I decayed at much lower levels over a 20-week period.

4. The antibody titer determined by PMI, using ELISA and RAC-L as the fixed antigen, showed that GF-II increased gradually post-immunization and surpassed that of RAC-L at 10 weeks and maintained higher values for the entire study period.

5. Antibody titer values determined by USAMRIID (using ELISA and ricin toxoid as the fixed antigen), beginning at week 6 through week 20.5, show a continuous rise in the IgG titer for GF-II, but continuous decline in the IgG titer values due to the commercial ricin A-chain (RAC).

6. The presence of an absorbable cation-exchanger in the gel-forming formulation (GF-II) contributes a successful controlled release of RAC and is associated with a different, and possibly, significant profile of antibody response.

7. Although the antibody titer values of GF-II exceeded those of RAC-L, the immunized mice with the latter system survived an intranasal challenge with ricin toxoid while those of the GF-II did not resist the challenge; such an expected result are attributed to alteration of the protective component of antigen during processing (dialysis, lyophilization, and drying) of RAC-L to incorporate it into the gel-former (GF-II).

8. A modified process for preparing GF-II with RAC needs to be developed to retain its protective component and maintain its proved ability to elicit high antibody response.

9. Intranasal immunization with ricin A-chain aqueous solution (RAC-L) cannot be recommended as it (1) was fatal to a fraction of mice shortly after immunization, and (2) provided limited protection when intranasally challenged with ricin toxoid. And, a controlled delivery system comprising a properly constituted (submicron anion- and cation-exchangers in a fluid, gel-forming, adhering matrix) is expected to be most effective.

10. Although a low viscosity gel-formulation (GF-III) did not appear to be suitable for intranasal immunization of mice, using RAC immobilized on the absorbable cation-exchanger; a much lower viscosity system with higher amounts of submicron ion-exchanger will be expected to be more efficacious for the immunization of animals larger than mice.

It is **recommended** that PMI will continue the optimization study of GF-II at no cost to the DoD over the next 5-6 months to eliminate the suspected alteration of the RAC antigenicity using a revised processing scheme for its incorporation in the gel-forming system, which will be further modified to accentuate the role of the micronized ion-exchanger. It is also **recommended** that one intranasal challenge study be conducted at the conclusion of the 5-6 month period at USAMRIID.

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Yan, C. et al, *Vaccine*, 13 (1995b) in press.

APPENDIX A

Outline of the Total Protein Assays Used for RAC *In Vitro* Release Study

Elisa Buffers

1)TBS

2.42 g Tris Base
8.0 g NaCl
Adjust pH to 7.6 with strong HCl (Slowly)
Make to 1 L (Distilled water)
Label "TBS"

2)Blocking Buffer

Take 250 ml of #1 and add:

1) 250 µl of Tween 20, 0.1%

2) 250 mg BSA (Albumin, Bovine (serum)) 0.1%

This is found in the little freezer underneath the DSC

3) 250 mg Powdered milk 0.1% Warm to 37° C for ± 30 min. to dissolve Label with contents and "Blocking Buffer"

3) TBS Tween 0.05%

Take 250 ml of #1

Add: 125 ul of Tween 20

Label "TBS Tween 0.05%"

1) Carbonate Buffer (To dissolve Ricin)

0.1 M:

1L water
1.36 g Sodium Carbonate
7.35 g Sodium Bicarbonate
pH to 9.4 (HCl)

OR Half batch numbers:

500 ml water
0.68 g Sodium Carbonate
3.675 g Sodium Bicarbonate

NOTE: Refrigerate all after making

Pierce Micro BCA Assay (Use for protein concentrations 0.5 to 20 ug/ml)

1. Working reagent is prepared by mixing two of the supplied solutions
2. One or two milliliter of sample and the same amount of working reagent are mixed well.
3. Tubes are mixed well and incubated at 60 C for 1 hour.
4. Cool to room temperature and measure the absorbance at 562 nm of each tube.

Standards are prepared from a suitable protein (BSA) and a curve is prepared after the samples are read.

Pierce BCA Assay (Use for protein concentrations 10 to 2,000 ug/ml)

1. Working reagent is prepared by mixing two of the supplied solutions
2. 0.1 milliliter of sample and 2.0 ml of working reagent are mixed well.
3. Tubes are mixed well and incubated at 60 C for 0.5 hour.
4. Cool to room temperature and measure the absorbance at 562 nm of each tube.

Standards are prepared from a suitable protein (BSA) and a curve is prepared after the samples are read.

APPENDIX B Animal Protocol for Mice Immunization

CLEMSON UNIVERSITY

PROTOCOL FOR USE OF LIVE VERTEBRATES

(SUBMIT THE ORIGINAL AND TWO COPIES TO THE ANIMAL RESEARCH COMMITTEE COORDINATOR, 301H BRACKETT HALL)

PRINCIPAL INVESTIGATOR Dr. Shalaby W. Shalaby **PHONE:** 646 - 8544

P.I. OF ACTIVITIES AT CLEMSON Dr. J. David Gangemi **PHONE:** 656 - 1440

COLLEGE/UNIT

DEPARTMENT Greenville Hospital System/Clemson University Biomedical Cooperative

SIGNATURES

As principal investigator, co-investigator, farm manager, or department head/chair, I verify that: (1) the information herein is true and correct and that I am familiar with and will comply with the legal standards of animal care and use established under federal laws, state laws and guidelines, as well as university policies; (2) the proposal has received approval for scientific merit by peer review; and (3) the activities do not unnecessarily duplicate previous experiments. I agree to advise the Animal Research Committee in writing of any changes in the procedures or personnel involved in this project. Such changes will not be implemented until committee approval is obtained. If a change in principal investigator becomes necessary, the committee will be notified immediately.

PRINCIPAL INVESTIGATOR S. W. Shalaby DATE: 5/10/96

FARM MANAGER DATE:

P.I. OF ACTIVITIES AT CLEMSON J. David Gangemi DATE: 5/13/96

DEPARTMENT CHAIR J. David Gangemi DATE: 5/13/96

Your signature as attending veterinarian verifies that you have: (1) reviewed and are familiar with this proposal, (2) consulted with the principal investigator(s) regarding any surgical procedures and any other procedures that may result in pain or distress, and (3) agree to perform all duties of the attending veterinarian in accordance with The Animal Welfare Act.

ATTENDING VETERINARIAN H E F Jr DATE: 5-13-96
(SIGNATURE)

TYPED NAME Harold E. Farris, Jr.

FOR ARC USE ONLY

ASSIGNED TO: ☐ FULL COMMITTEE ☒ DELEGATED

AUP NUMBER 96-035

USDA CATEGORY

AMENDMENT NUMBER, IF APPLICABLE 1

INSTITUTIONAL CATEGORY

ARC APPROVAL: Elizabeth Kunkel 6-4-96

CHAIR SIGNATURE DATE:

CLEMSON UNIVERSITY

PROTOCOL FOR USE OF LIVE VERTEBRATES

(SUBMIT THE ORIGINAL AND TWO COPIES TO THE ANIMAL RESEARCH COMMITTEE COORDINATOR, 301H BRACKETT HALL)

PRINCIPAL INVESTIGATOR Dr. Shalaby W. Shalaby **PHONE:** 646 - 8544

P.I. OF ACTIVITIES AT CLEMSON Dr. J. David Gangemi **PHONE:** 656 - 1440

COLLEGE/UNIT _____

DEPARTMENT Greenville Hospital System/Clemson University Biomedical Cooperative

SIGNATURES

As principal investigator, co-investigator, farm manager, or department head/chair, I verify that: (1) the information herein is true and correct and that I am familiar with and will comply with the legal standards of animal care and use established under federal laws, state laws and guidelines, as well as university policies; (2) the proposal has received approval for scientific merit by peer review; and (3) the activities do not unnecessarily duplicate previous experiments. I agree to advise the Animal Research Committee in writing of any changes in the procedures or personnel involved in this project. Such changes will not be implemented until committee approval is obtained. If a change in principal investigator becomes necessary, the committee will be notified immediately.

PRINCIPAL INVESTIGATOR _____ DATE: _____

FARM MANAGER _____ DATE: _____

P.I. OF ACTIVITIES AT CLEMSON _____ DATE: _____

DEPARTMENT CHAIR _____ DATE: _____

Your signature as attending veterinarian verifies that you have: (1) reviewed and are familiar with this proposal, (2) consulted with the principal investigator(s) regarding any surgical procedures and any other procedures that may result in pain or distress, and (3) agree to perform all duties of the attending veterinarian in accordance with The Animal Welfare Act.

ATTENDING VETERINARIAN _____ DATE:

(SIGNATURE)

TYPED NAME Harold E. Farris, Jr.

FOR ARC USE ONLY

ASSIGNED TO: ☐ FULL COMMITTEE ☐ DELEGATED **AUP NUMBER** _____

USDA CATEGORY _____ **AMENDMENT NUMBER, IF APPLICABLE** _____

INSTITUTIONAL CATEGORY

ARC APPROVAL:

CHAIR SIGNATURE DATE:

GENERAL INFORMATION

1. SUBMISSION TYPE

New
X **Addendum**
Renewal/Continuation (Previous Protocol Number _____)

2. CLASSIFICATION

X Research
Teaching
Demonstration
Farm Management

3. FUNDING SOURCE(S) & GRANT APPLICATION TITLE(S), IF APPLICABLE

U. S. Department of Defense Small Business Innovation Research Program

4. COURSE NUMBER(S) AND TITLE(S), IF APPLICABLE

N/A

5. PROJECTED START DATE March 15, 1996 **PROJECTED END DATE** September 15, 1996

6. ABSTRACT

PHS policy requires submission of an abstract including the items listed below. The abstract must be submitted as Attachment 1 and should not exceed two (2) pages. **The abstract must be written to ensure comprehension by non-scientists** (preferably at high-school level). The following must be included:

- objectives of the research or teaching activity
- species and number of animals
- schedule of the course or the study procedures performed during each phase
- benefits, outcome and results expected

7. FLOW SHEET

A flow sheet is required and must be submitted as Attachment 2. Relative information (i.e., numbers of animals, experimental manipulations) should be provided on a flow sheet diagram. The ARC member should be able to follow each manipulation of the animal from initiation to termination.

8. PHS POLICY

PHS policy requires a copy of *Section F from NIH applications* and a copy of all animal methods sections from the proposal. Please provide as an attachment, **if applicable**. DO NOT SUBMIT THE ENTIRE APPLICATION.

GENERAL INFORMATION
DUPLICATE PAGE AS NEEDED

9. DESCRIPTION OF ANIMALS (*COMMON NAMES REQUIRED*)

SPECIES: **Mice**		
BREED/STRAIN: **NIH Swiss**		
X **GROUP HOUSING** SINGLE HOUSING	IF GROUP, NO. PER CAGE: **3/cage**	
MALE **X** **FEMALE**	AGE: **6-8 wks.**	WEIGHT: **20 -23 g**
Maximum number maintained at any given time	Total Number Required	Comments
88 mice	***149 mice***	

SPECIES:		
BREED/STRAIN:		
GROUP HOUSING SINGLE HOUSING	IF GROUP, NO. PER CAGE	
MALE FEMALE	AGE	WEIGHT
Maximum number maintained at any given time	Total Number Required	Comments

SPECIES:		
BREED/STRAIN:		
GROUP HOUSING SINGLE HOUSING	IF GROUP, NO. PER CAGE	
MALE FEMALE	AGE	WEIGHT
Maximum number maintained at any given time	Total Number Required	Comments

GENERAL INFORMATION

10. SOURCE OF ANIMALS

X **Commercial Vendor** Charles River Company, Brookline, Massachusetts
Captured from Wild (A copy of the permit MUST be attached)
Transferred from Another Protocol; provide number_____
Bred or reared at Clemson
Donated to become Clemson property
Other: _____

11. METHOD OF ANIMAL IDENTIFICATION

X **Cage Cards**
X **Ear-Punch**
Collars
Leg Bands
Wing Tags
Other Tags
Tattoos
Electronic
Branding (Freeze)
Branding (Hot-Iron)
Other _____

12. DISPOSAL OF ANIMALS AFTER COMPLETION OF STUDY/PROCEDURE

X **Euthanized by methods outlined in the Euthanasia section of this protocol**
Returned to wild
Returned to production/breeding unit
Sold
X **Transferred protocol number:U.S. Army Medical Research Institute of Infectious Diseases**
Slaughter with conformation to the Humane Slaughter Act
Other:_____

ALTERNATIVES TO THE USE OF ANIMALS - The Animal Welfare Act requires that the Animal Research Committee must ensure the principal investigator has considered alternatives to procedures that may cause more than momentary or slight pain or distress to animals, and has provided a written narrative description of the methods and sources.

13. What is the justification for using live animals rather than alternative means of achieving the research goals?

There is no *in-vitro* model to substitute for the *in-vivo* one.

GENERAL INFORMATION

14. You are required to conduct a literature search to determine that either (1) there are no alternative methodologies by which to conduct this study, or (2) there are alternative methodologies, but these are not appropriate for your particular study. What procedures, specific databases, and sources did you use to determine that non-painful alternatives were not available or appropriate?

AGRICOLA
Animal Welfare Information Center
Biological Abstracts
Index Medicus
National Agricultural Library *Phone: (301) 504-6212*
Contacted colleagues
X **Other** **MED-LINE, Chem Abstracts, National Medical Library**

What were your findings with respect to alternative methodologies?

No alternatives exist.

15. Why have you selected the particular species proposed in this project?

Lowest form of vertebrate.
All previous work on similar compunds used mice.

16. Provide an explanation of how the numbers of animals to be used were derived. Numbers should be based on scientific and statistical requirements to achieve objectives. These numbers must be consistent with those used in the flowsheet. If statistical assistance is needed, contact the Office of Experimental Statistics at 656-3028.

It was previously established for the antibody study that five mice is the minimum, and for the challenge study, ten mice is the minimum required.

Yan et al., *Vaccine* 13, 645 (1995).
Yan et al., *Journal of Controlled Release* 32, 231 (1994).

DUPLICATION OF RESEARCH - The Animal Welfare Act requires that the principal investigator provide written assurance that proposed research is not unnecessarily duplicative.

17. Does the proposed research duplicate any previous work?

Yes
X **No**

17a. If **no**, what procedures and sources did you use to determine that the proposed research does not duplicate previous work?

MED-LINE, Chem Abstacts, and the National Medical Library

17b. If **yes,** provide justification for the need to duplicate previous work.

QUALIFICATIONS OF PERSONNEL
(Duplicate this page as needed.)

Carefully review the Qualifications of Personnel instruction section located in the front of this form. Complete the following for people who will conduct procedures using animals (especially surgery, anesthesia, pre- or post-operative care, or euthanasia). An occupational health program is mandatory for personnel who work with laboratory animal facilities or have animal contact. The ARC will provide advice and training on all of these procedures if the personnel listed have no previous relevant experience..

List individuals who will have animal contact in association with this protocol and provide the date of their enrollment the Clemson University occupational health program. If individuals listed in this section are not enrolled, they must contact the Nursing and Wellness Center at 656-3076 to enroll or to sign a waiver of exclusion. Individuals not enrolled cannot participate in the study and approval will not be given until enrollment is verified.

FULL NAME: **Research Services Personnel**

TITLE: _____

Clemson University Animal Care and Use Seminar attendance

¢ Date attended: _____ **or**
¢ Date scheduled to attend: _____

Occupational Health/Medical Surveillance Enrollment

¢ Date of Enrollment: _____ **or**
¢ Date Waiver Signed: _____

Procedure(s) being performed by this individual. Check all applicable procedures.

¢ X **Injections**
¢ X **Feeding**
¢ X **Weighing**
¢ X **Blood Collection**
¢ X **Euthanasia**
¢ Surgery
¢ Oral Gavage
¢ X **Other, specify: Ear punch**

Qualifications/relevant experience. Indicate if this individual has performed the procedure with this species previously?

Qualifications on file at Research Services office.

EUTHANASIA

[THE ATTENDING VETERINARIAN MUST BE CONSULTED REGARDING THE METHOD OF EUTHANASIA PRIOR TO SUBMISSION OF PROTOCOL.]

This section must be completed for every protocol; even though your study does not involve planned euthanasia. The method outlined may be used in the event of unanticipated injury or illness. Following euthanasia, death should be assured by creating a bilateral pneumothorax, aortic transection, or other certain physical means as appropriate.

INDIVIDUAL(S) PERFORMING EUTHANASIA:

Name **Research Services Personnel**

Name _____

Name _____

Name _____

POSSIBLE METHOD	SPECIES 1	SPECIES 2	SPECIES 3
CO_2 Precharged Chamber	X		
Cervical Dislocation under CO_2 anesthesia			
Decapitation under CO_2 anesthesia			
Captive Bolt			
Cervical Dislocation *			
Decapitation *			
Injectable euthanasia agents (see dose chart below)			
Other *			

* Must include justification if the method is not recommended by the AVMA Panel on Euthanasia.

Species	Agent	Dose	Route
NIH Swiss mice	CO_2	Saturated Chamber	Inhalant

USE OF ANIMALS IN BIOMEDICAL AND AGRICULTURAL RESEARCH AND TEACHING

*** COMPLETE THOSE SECTIONS RELEVANT TO YOUR PROJECT - DISCARD ALL SECTIONS NOT APPLICABLE TO YOUR PROJECT ***

CHECK APPLICABLE SECTIONS:

¢	X	SECTION A	**Non-Surgical Procedures Involved** Experimental procedures, including non-surgical, pre-surgical, and post-surgical.
¢	X	SECTION A	**Antibody Production Involved**
¢		SECTION B	**Surgical Procedures Involved** Non-Survival Surgery Survival Surgery Multiple Survival Surgery
¢		SECTION C	**Field Studies Involved**
¢	X	SECTION D	**Hazardous Agents Involved** Copy of approval letter from the Institutional Biosafety Committee must be submitted prior to ARC approval.
¢		SECTION E	**Farm Animals Used in Agricultural Teaching and Production**

SECTION A
NON-SURGICAL PROCEDURES

1. SITE OF PROCEDURE(S)

BUILDING **Godley Snell Research Center**
ROOM _____

2. CHECK THE FOLLOWING ITEMS APPLICABLE TO YOUR STUDY AND COMPLETE APPROPRIATE SECTIONS.

- X **Antibody Production**
- X **Blood Withdrawal**
- Restraint with mechanical devices
- X **Nutritional deficiencies, tumor and disease models or toxicity testing**
- X **Anesthesia or analgesia**

ANTIBODY PRODUCTION - Describe procedure, antigen and adjuvants used, the ratio of antigen to adjuvant and routes of injection: (volume should be less than 0.2 ml per injection site; subcutaneous route is recommended.)

0.2 ml gel formulation or placebo administered via subcutaneous injection in Segment I of study.
50 μl gel formulation or placebo administered via intranasal injection, 25μl/nostril in Segment II of study.

BLOOD WITHDRAWAL - Describe method(s), volume(s) collected and frequency of collection.

0.2 – 0.5 ml blood withdrawn from retro-orbital sinus at 4, 6, 8, and 10 wks.

RESTRAINT WITH MECHANICAL DEVICES - Describe device, duration of restraint, conditioning procedures and steps to assure comfort and well-being of animal.

N/A

List non-surgical procedures for which anesthetics, analgesic or tranquilizers are required.

Procedure	Drug	Dose	Route
Ear Punch	isoflurane/ O_2	precharged chamber for induction (4 -5 % in 2 - 3 l/m O_2) then maintaenance via mask at 2-3 %	inhalant
	OR 1.75 ml ketamine 0.75 ml xylazine 2.5 ml sterile water	0.1 ml for 20 g mouse or 0.12 ml for mouse of 25 g or more	intraperitoneal injection
retro-orbital bleeding	same as for ear punch		
intranasal injection	same as for ear punch		

SECTION A
NON-SURGICAL PROCEDURES

PROCEDURES THAT MAY INDUCE MODERATE TO SIGNIFICANT PAIN, DISTRESS, OR DISCOMFORT, FOR EXAMPLE, NUTRITIONAL DEFICIENCIES/ASCITES TUMOR, INFECTIOUS DISEASE MODELS; NEOPLASIA MODELS; TOXICITY TESTING - Describe methodology. State objective criteria used to assess health, pain and distress during course of study. Include clinical signs or manifestations expected from the procedure. What criteria will be used to determine a humane endpoint before severe morbidity and death?

Gel formulations or placebo administered via subcutaneous injection in Segment I and via intranasal injection in Segment II.
No expected clinical toxicity.
Humane end-point defined as 20% weight loss.

	HEALTH ASSESSMENT	Observation Frequency (2x Daily, Daily, 2x Weekly, Weekly)
X	**Body Temperature**	**2 times weekly if other parameters are abnormal**
X	**Weight**	**2 times weekly or more frequently if other parameters are abnormal**
X	**Behavior**	**daily**
X	**Other: general appearance (hair coat, hydration status, activity) appetite**	**daily**

	HUMANE ENDPOINTS If these humane endpoints are not appropriate for the study or cannot be used for scientific reasons, scientific justification must be provided for ARC review and inclusion in the USDA Annual Report of Research Facilities.
X	**Ataxia**
X	**Depression > 48 hours**
	Infection
X	**Weight loss of 20% as compared to normal growth charts**
	Non weight bearing > 72 hours
X	**Abnormal vocalization**
	Inappetence > 48 hours
X	**Inactivity**
X	**Reluctance to move**
	Other behavioral, physiological, or biochemical criteria - specify:

SECTION D
HAZARDOUS AGENTS

IF ANY PROJECT INVOLVES USE OF THE FOLLOWING HAZARDOUS AGENTS, AN APPLICABLE BIOHAZARD PROTOCOL MUST FIRST BE APPROVED BY THE INSTITUTIONAL BIOSAFETY COMMITTEE BEFORE FINAL APPROVAL OF THE ANIMAL USE PROTOCOL IS GRANTED. **A COPY OF THE IBC APPROVAL WITH APPROVED PROTOCOL NUMBER AND IBC CHAIR SIGNATURE MUST BE ATTACHED.**

1. WILL THIS PROJECT REQUIRE THE USE OF IONIZING RADIATION?

Yes
X No

If yes, list isotope(s) and/or describe irradiation procedure.

2. WILL THIS PROJECT REQUIRE THE USE OF HAZARDOUS BIOLOGIC AGENTS (HUMAN/ANIMAL PATHOGENS, TUMOR CELLS) OR RECOMBINANT DNA?

Yes
X No

If yes, list agent(s) and classification.

N/A

If tumor cells will be used, have they been tested for contamination by viruses?

N/A Yes
No

3. WILL THIS PROJECT INVOLVE USE OF TOXIC CHEMICALS OR CARCINOGENS?

X Yes
No

If yes, please describe or list.

Ricin A Toxoid

ABSTRACT

Phase I objective is to determine feasibility of using novel, absorbable liquid gel formers in developing injectable single-shot vaccine formulations, which provide timely antibody response and prolonged immunity. Phase I study includes (1) review of available in-house data, that are being generated, on performance of novel gel formers in controlled release of antibiotics, polypeptides, and bovine serum albumin and select suitable compositions for vaccine formulation using ricin A toxoid (RT) vaccine as a model; (2) synthesis and characterization of selected polymeric compositions; (3) aseptic preparation and characterization of needed formulations; (4) determination of in-vitro release profile of different formulations; (5) determination of vaccine stability in key formulations; (6) completion of an immunization study in mice and determination of antibody response; and (7) use of immunized mice to assess the persistence of protection against an aerosol challenge with RT. Most pertinent to this protocol is a description of the intended animal studies which can be documented as follows.

Using lyophilized RT provided by USAMRIID, the immunization of mice will be conducted as described by Yan and coworkers (1995b). Each formulation will be used in seven mice, five for blood analysis and two spare mice. For immunization, female, 6-8 week-old NIH-Swiss mice will be purchased. The mice will be housed in bioclean cages and fed and watered *ad libitum*. For the Segment I Animal Study, RT/GF, RT/GF/MC, RT-solution, or placebo GF/MC will be administered by subcutaneous inoculation as indicated in Table I. For the Segment II Animal Study, RT/GF/MC, RT-solution, or placebo GF/MC will be administered by intranasal inoculation as indicated in Table II. For each case, the interval between multiple doses of the control RT-solution is three weeks.

For each active gel formulation and the active control (using RT solution) samples, a set of 10 mice will be immunized as described above. Based on the results of the *in-vitro* release studies and the antibody titer determination, the post-immunization periods preceding the challenge will be determined, which may be different from the 4 and 6 weeks tentatively noted in Tables I and II. At the predetermined period, the immunized mice will be challenged by whole-body exposure to 100 mg/kg ricin toxin delivered by aerosol. Groups of up to 50 mice will be exposed to ricin in a dynamic, whole-body exposure chamber with a total system airflow rate of 19.5 liters per minute (LPM). A small-particle aerosol, with a mass median aerodynamic diameter of 1.2 mm, will be produced by a collision nebulizer. The aerosol will be sampled during the entire exposure period of 10 min by using a standard all-glass impinger containing 10 ml of sterile PBS, pH 7.4. Ricin toxin in the collection fluid will be measured by protein analysis (Pierce Micro BCA, Pierce Labs., Rockford, Illinois). Respiratory minute volumes will be estimated by using Guyton's formula, which is based on animal weight (Guyton, 1947). An estimated inhaled dose will be calculated from the respiratory breathing rates and the aerosol concentration of toxin delivered. Exposed mice will be observed daily for 3 - 4 weeks. Statistical significance will be calculated by Fisher's two-tailed test.

Phase I primary outcome is demonstrating feasibility of using liquid gel formers in single-shot injectable vaccine formulations for prolonged immunity. Phase I data will be used to determine the applicability of the carrier system for one-dose delivery of vaccines and complete development and scale-up studies of a selected formulation. Short-term impact will be production of a one-dose vaccine formulation of interest to the military. Extension of the gel technology to known labile and new vaccines and development of one-dose, oral vaccines will be long-term medical and commercial pay backs.

Experimental Schemes for Segment I Animal Sudy

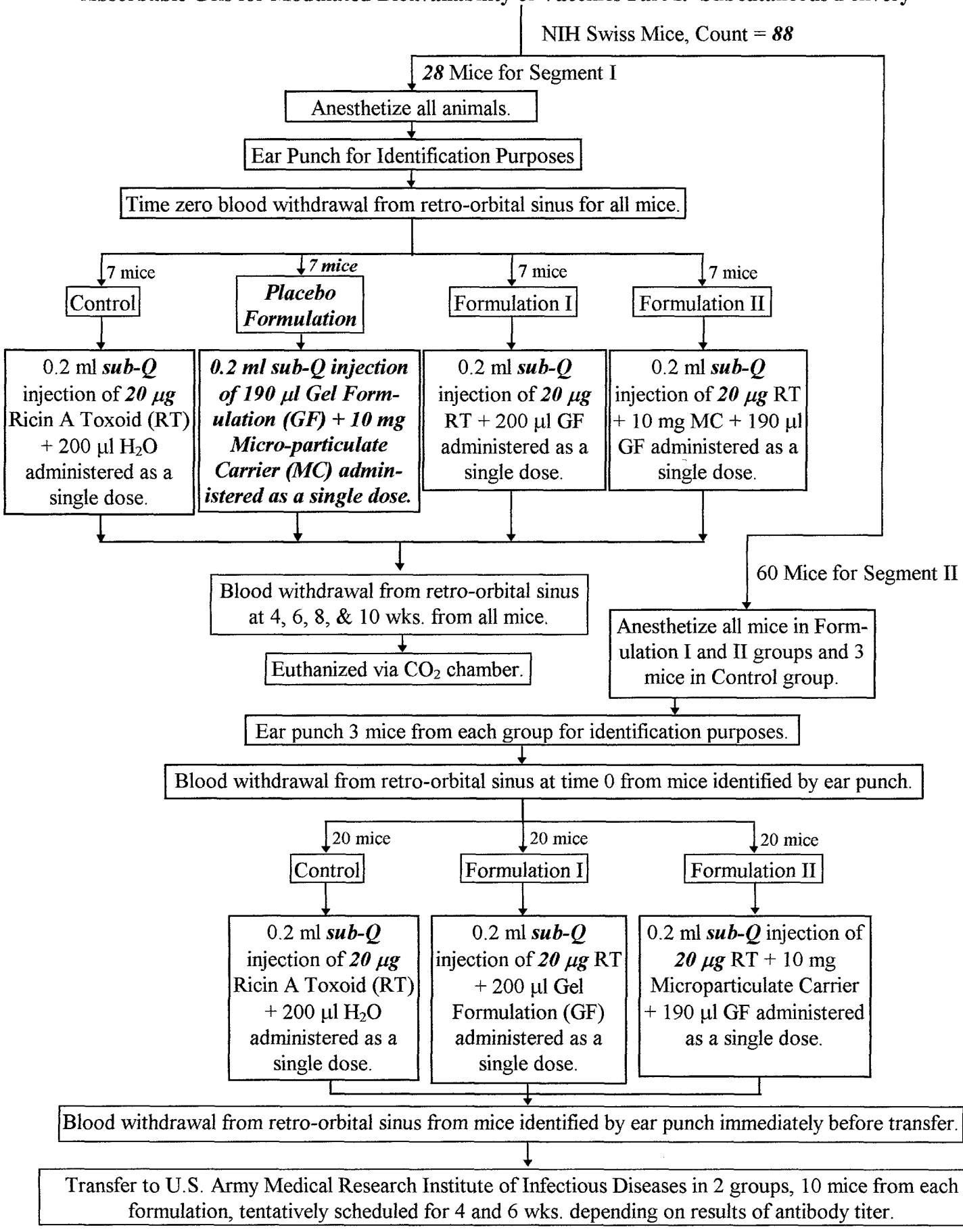
SEGMENTS	CONTROL	PLACEBO I	FORMULATION I	FORMULATION II
Formulation for Subcutaneous Administration				
• Polymer	None	190 µl Gel-former (GF) + 10 mg Microparticulate carrier (MC)	200 µl GF	190 µl GF + 10 mg MC
•Ricin Toxoid (RT)	20 µg RT + 200 µl H_2O	None	20 µg RT	20 µg RT
Determination of antibody titer (a)	← 0, 4, 6, 8 & 10 wks. →			
Immunization and Persistence of Protection (b)				
•Determination of antibody titer	immediately prior to transfer for challenge	N/A	immediately prior to transfer for challenge	immediately prior to transfer for challenge
•Post-immunization challenge (c)	5 to 7 wks. & over 7 wks.	N/A	5 to 7 wks. & over 7 wks.	5 to 7 wks. & over 7 wks.

Experimental Schemes for Segment II Animal Studies

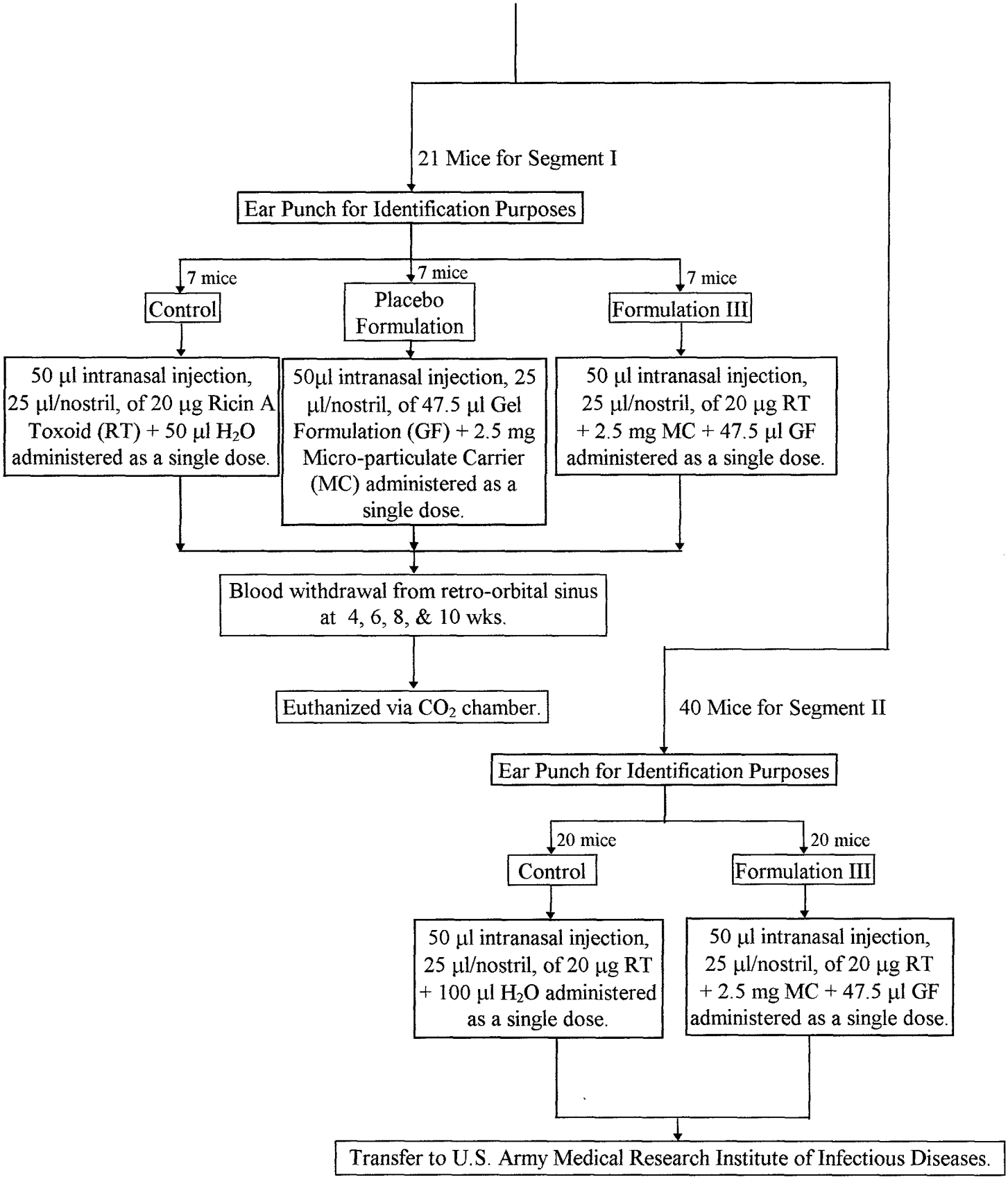
SEGMENTS	CONTROL	PLACEBO II	FORMULATION III
Formulation for Intranasal Administration			
• Polymer	None	47.5 µl GF + 2.5 mg MC	47.5 µl GF + 2.5 mg MC
• RT	20 µg RT + 200 µl H_2O	None	20 µg RT
Determination of Antibody Titer (a)	← 0, 4, 6, 8 & 10 wks. →		
Immunization and Persistence of Protection (b)			
• Determination of antibody titer	immediately prior to transfer for challenge	N/A	immediately prior to transfer for challenge
•Post-immunization challenge (c)	5 to 7 wks. & over 7 wks.	N/A	5 to 7 wks. & over 7 wks.

(a) Each experiment will be run in a set of seven animals.
(b) Each experiment will be run in a set of ten animals.
(c) Post immunization challenge will be conducted at Fort Detrick.

Absorbable Gels for Modulated Bioavailability of Vaccines Part I: Subcutaneous Delivery



Absorbable Gels for Modulated Bioavailability of Vaccines
Part II: Intranasal Delivery
NIH Swiss Mice, Count = 61



APPENDIX C

SOP of Elisa Assay for Anti-RAC Antibodies

Pates: 96 well microtiter plates

Day 1:

Add 50 ul RAC @ 50 ug/ ml in carbonate buffer to each well
Place in 37 C shaker for 3 hours
Wash 3 X with 100ul TBS/ Tween 0.05%
Add 200 ul of blocking solution (BSA and powdered milk)
Block for 1 hour at 37 C
Wash 3x with 100 ul TBS/ Tween 0.05%
Add primary antibody dilutions, titer dilutions, etc.
Incubate overnight (15 hours) in refrigerator

Day 2:

Wash 3x with 100ul TBS/Tween 0.05%
Add 50 ul of 1:2000 (in TBS/ Tween 0.05%) anti-mouse antibody conjugate
Incubate for 2 hours at 37 C
Wash 4x with TBS/ Tween 0.05%
Add 50 ul of PNPP solution
Read absorbance of each well at 405 nm.

APPENDIX D

List of Personnel at Poly-Med, Inc., Receiving Pay for This Project.

Joel T. Corbett
Jacqueline M. Allan
Valerie J. Fracault
Russell A. Johnson
Shalaby W. Shalaby

CONTRACT NO.: DAMD 17-96-C-6045

Absorbable Gels for Modulated Bioavailability of Vaccines

TYPE OF REPORT: Final Report Addendum
Covering September 15 - November 8, 1996

CONTRACTOR: Poly-Med, Inc.
6309 Highway 187
Anderson, South Carolina 29625

Phone: (864) 646-8544
Fax: (864) 646-8547

PRINCIPAL INVESTIGATOR: Shalaby W. Shalaby, Ph.D.

DATE OF PUBLICATION: November 8, 1996

CONTRACTING OFFICER'S REPRESENTATIVE: M. Kende, Ph.D.

Section I. Introduction

The funded segment of Phase I study of this contract was completed on September 15, 1996, and the final report was issued on October 14, 1996. Meanwhile, Poly-Med was granted an extension of the study, without additional funding until October 26, 1996. Results associated with the unfunded period are addressed in this addendum to the final report. Tentative plans for additional unfunded studies are also included. Pursuing the latter is contingent upon USAMRIID approval to conduct the aerosol challenge of immunized mice, if so needed (in the event the ricin neutralization assay is shown to be inconclusive) at Fort Detrick.

Section II. Results of the Extended Segment of Phase I

The aerosol challenge of the subcutaneously immunized mice at 24 weeks was conducted. The results were similar to those reported at 20.5 weeks in the final report. In addition, the aerosol challenge of the intranasally immunized mice at 16 weeks was conducted and results were also similar to those documented in the final report for 12.5 weeks post-immunization. A brief 4-week study was conducted with RAC-L and reconstituted RAC that has been purified and lyophilized earlier. Three mice were used in each of the sets. The serum antibody titer values (using ELISA) at 3-week post-immunization were low for both sets of mice and no significant difference between them could be determined. This was followed by an aerosol challenge of both sets of mice with ricin toxoid (at Fort Deterick) at the conclusion of the 4-week post-immunization period. Results indicate that both sets of mice displayed partial protection.

Section III. Follow-up on the Phase I Recommendations

As a follow-up of the Phase I study, it is recommended that the following probing study be conducted without additional funding, should the USAMRIID agree to conduct the antibody neutralization on immunized mice sera or the aerosol challenge study on the immunized mice. This study is intended to determine the cause of compromising the protective capacity of RAC in the controlled delivery system while displaying substantial antibody response, as determined by ELISA.

A. Immunization and Antibody Titer Determination at Poly-Med

Four sets of seven animals will be subcutaneously immunized with the following formulation described in Table I, and their antibody titer will be determined at 10 and 14 weeks.

Table I. Formulations for the Probing Study

	Composition of Formulation*			
Set No.	A	B	C	D
Type of RAC	Commercial Solution**	Purified/lyophilized/ reconstituted	Same as B	Commercial Solution
Cation Exchange mg	None	10	20	10
Gel Former II μl	None	190	180	190

* All mice will be injected subcutaneously with 0.2 ml. of formulations which contain 20 μg of RAC. All components will be micromixed with no further treatment and stored at 4°C.

** Using diluted solution to attain 0.2 ml volume.

B. Evaluation of Protection Level at USAMRIID

These evaluations will be conducted at the end of the 14- or 16-week post-immunization period. The evaluation will entail an aerosol challenge of the immunized mice at 16 weeks, if the ricin neutralization assay with immune serum at 14 weeks is found to be inconclusive.

C. Expected Results and Value for Future Work

Results of the probing study are expected to provide us with (1) an insight as to the cause of discrepancies between ELISA and aerosol challenge data; (2) additional data on the role of the cation-exchanger; and (3) information as to the effect of the RAC processing method on its immunizing capacity.

Section IV. Administrative Comments

Timely approval by USAMRIID of the proposed collaborative effort in Section III-B is crucial for a prompt initiation and successful completion of the study.

In the event that PMI is awarded Phase II contract, results of the "Probing Study" will be used to streamline the planned activities for that phase of the program. In turn, this will increase the effectiveness of Phase II experimental efforts and impact, positively, its practical outcome.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 4 Dec 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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